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A robust micropropagation protocol for genetically true to type plants of Phule Arakta pomegranate

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ABSTRACT

A rapid and reproducible micropropagation protocol for pomegranate cv. Phule Arakta has been developed using nodal segments of field grown plant. Bud break was induced in basal Woody Plant Medium (WPM) as well as compared when WPM was supplemented with plant growth regulators. Multiple shoot proliferation was induced in the nodal segments on WPM fortified with different concentration of benzyladenine (BAP) where 2mg/l BAP developed maximum number of shoots. Elongation of shoots was further amplified with the addition of adjuvant silver nitrate. Browning of culture medium was controlled by the addition of polyvinylpyrrolidone (PVP) and regular sub-culturing enhanced shoot multiplication as well as elongation. Rooting was induced in the regenerated shoots using Indole-3-butyric acid (IBA) and 3-Indoleacetic acid where best result was obtained using shock treatment with NAA. Sub-culturing resulted in denser and better rooting. The rooted plantlets were further acclimatized and then established in soil. The clonal fidelity of the *in vitro* grown cultures was assessed using Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) markers. The 10 RAPD decamers produced 55 bands and 4 ISSR produced 19 bands in total. RAPD primers OPC-08, OPC-13 and OPD-07 produced the highest number of distinct bands and ISSR primer UBC-834 produced maximum distinct bands. All the bands were monomorphic which confirms the genetic fidelity of the *in-vitro* raised *P. granatum* cv. Phule Arakta and supported the method of mass production of true to type progenies using tissue culture.

Keywords: *Punica granatum*, genetic fidelity, RAPD, ISSR.

INTRODUCTION

Punica granatum L. (Pomegranate) belongs to the *Lythraceae* family and is one of the oldest known edible fruit trees (Noormohammadi *et al.*, 11). It comprises only one genus –*Punica* and two species- *P. granatum* and *P. protopunica* (Samir, 13). This fruit has high nutritional and medicinal value and is native to Central Asia, Iran, Afghanistan, Pakistan and Himalayas in Northern India (Patil *et al.*, 12). It is now commonly cultivated in many areas all over the world.

In India, Maharashtra is the leading producer of pomegranate followed by other states such as Karnataka, Gujarat, Andhra Pradesh, Madhya Pradesh and Tamil Nadu (Shukla *et al.*, 14). Due to its high market value with excellent health benefiting properties, pomegranate is an excellent choice for growing in a large scale for income generation. The cultivar "Phule arakta" possesses desirable traits such as high yield, large size and sweet to taste with glossy dark red skin, and is commercially cultivated in various regions of Maharashtra. Traditionally, it has been grown through seeds in the field which results in genetic variation and through cutting and grafting which is labor intensive, time bound and does not ensure disease free plant. Tissue culture techniques have been widely employed in recent years for obtaining plantlets for the mass propagation of important tree species (Akin *et al*., 2; Kovalchuk *et al.*, 7). Another important application involves the regeneration of genetically transformed plants for better traits.

In recent years, apart from obtaining large quantity of disease free plants, tissue cultured plants are also assessed for their clonal uniformity using various molecular markers such as Randomly Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR), Inter-simple sequence repeats (ISSR), etc. RAPD is considered as a cost effective and simple technique to detect variations. ISSRs does not require any pre genome sequence information, with low production cost and easily applicability to any plant species. The most important application of ISSR is its high reproducibility and ability to generate ample polymorphisms, which have been successfully used to differentiate individuals that are closely related. (Abate, 1).

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Several researchers have also reported micropropagation protocol for other pomegranate cultivars using different explant sources (Naik *et al*., 10; Patil *et al*., 12; Valizadeh Kaji *et al*., 16); however, only few micropropagation protocols with genetic fidelity testing have been reported (Guranna *et al*., 6). Therefore, the present study was taken to develop an efficient and reproducible micropropagation protocol of this important cultivar and to check for their genetic uniformity in progenies using RAPD and ISSR markers before their release for large scale plantation.

MATERIALS AND METHODS

Plants were obtained from nurseries and maintained in the open field at Amity organic farm, Amity University, Noida, India, to be used as source of explant. Axillary branches were cut from the field grown mother plant and washed under running tap water after which leaves were excised and cut into 2cm pieces, each containing a nodal segment. This was followed by washing with 2-3 drops of Tween 20 (HiMedia Laboratories Pvt. Ltd.) and 70% ethanol for 30 seconds. They were then disinfected with 0.1% Sodium hypochlorite (Titan Biotech Ltd) for 10 minutes under sterile conditions inside the laminar air flow hood. Each of this disinfection step was followed by rinsing thrice with sterile distilled water after which, the ends of the surface sterilized nodal segments were trimmed and inoculated vertically onto induction medium (one explant per test-tube).

Woody Plant Media (WPM), Murashige and Skoog media (MS), Schenk & Hildebrandt media (SH) and Gamborg's B5 media were prepared using stock solutions made from individual chemicals in autoclaved distilled water, properly dissolved and labelled. 30g/l sucrose and 0.1g/l myo-inositol were added, and pH was maintained at 5.8±0.2 using 0.1N NaOH and/or 0.1N HCl before gelling with 0.7% agar powder. The prepared media was then autoclaved at 15psi, 121°C for 20 minutes. All the chemicals used were of analytical grade (HiMedia Laboratory Pvt. Ltd., Mumbai; Titan Biotech Ltd., Delhi). Explants were inoculated onto the different tissue culture media, maintained in sterile condition at 25°C under a 16 hours light (60µmols/m^2/s^2) and 8 hours dark photoperiod and checked for their effect on the bud break.

For obtaining shoot proliferation, BAP at different concentration (0, 1, 2 and 3 mg/l) was employed. Combination of different adjuvants such as silver nitrate, adenine sulphate and spermidine at varying concentration (0, 0.5, 1 and 1.5 mg/l) was also checked for their effect (Table 2). In order to overcome the problem of leaching and browning of culture media, PVP 250 mg/l was added into the media and poured into test-tubes and jars. Sub-culturing was carried out every 4 weeks by excising the shoots into nodal segments and culturing on the same media in jars. Readings of shoot number, shoot length, number of nodes and leaf number were noted down (Mean of each reading per explant). Each experiment consisted of five replicates repeated thrice.

In-vitro elongated shoots tips of 3-5cm were dipped into 100mg/l NAA and 100mg/l IBA for few seconds and transferred onto basal WPM media. The base of the test-tubes was wrapped with silver foil to create a dark environment which enhances root formation. Sub-culturing was carried out 2 weeks after rooting was obtained and then transferred onto same rooting media in a jar to provide more space. The root number and root length were noted after 4 weeks of sub-culturing.

Explants that were well rooted and had sturdy roots were removed from the media and washed gently under running tap water to completely remove any agar adhering to it. They were then transferred to small plastic pots containing autoclaved substrates such as cocoa peat, soilrite, vermiculite and pearlite. The pots were then maintained in plant growth chamber with artificial light provided by white fluorescent tubes (60µmols/m^2/s^2) and conditions of 55% humidity and temperature 28°C for 4-6 weeks.

The data were statistically analyzed using one way or two way analysis of variance (ANOVA) and the significance value was taken at α=0.05.

DNA from mother plant and progenies regenerated *in-vitro* from 3rd sub-culture, 6th sub-culture, randomly selected samples and rooted culture were isolated using the CTAB extraction protocol developed by Doyle and Doyle with some modifications that included RNaseA (HIMEDIA Laboratory Pvt. Ltd.) treatment before extraction using chloroform isoamyl alcohol. Mortar and pestle, micro-tips, pipettes and Eppendorf tubes were autoclaved before starting the experiment. For qualitative analysis, the sample DNA isolated was run on 0.7% agarose gel at 100 V to check the presence and size of DNA. Spectrophotometrically, qualitative and quantitative analysis of DNA was done by recording the optical density at 260 nm and 280 nm. A ratio of 1.8 ($O.D_{260}/O.D_{280}$) indicates very pure DNA. The DNA concentration was calculated by-Concentration of DNA= $O.D_{260}$ × Dilution factor × 50ng/ml.

Initially, 10 RAPD and 5 ISSR primers which were earlier reported for genetic fidelity testing of banana (Lakshmanan *et al*., 9) were screened for their

reproducibility in *P. granatum* L. cv "Phule arakta". PCR was carried out in a volume of 25µl containing 1µl DNA template (50 ng DNA), 2.5µl RAPD primer, 12.5µl PCR mix (OnePCR™, GeneDirex, Cat. No. MB203-0100) and remaining volume was made up with nuclease free water. PCR amplification was performed using PCR Thermocycler (Eppendorf Vapo.protect). For RAPD primers, initial denaturation was carried out at 94°C for 4 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, 36° C for 1 minute and 72°C for 2 minutes, with final extension at 72°C for 10 minutes. For ISSR primers, initial denaturation was carried out at 95°C for 2 minutes followed by 35 cycles comprising denaturation for 50 seconds at 94°C, annealing for 1 minute at 46°C±8°C and extension at 72°C for 50 seconds. The final extension was carried out for 15 minutes at 72°C.

The amplified PCR products were electrophoresed on 1.8% agarose gel with 0.5µ/ml Ethidium bromide solution and run in 1X Tris borate EDTA (TBE) buffer. A 1kb and 100bp DNA ladder marker was used to estimate the fragment size for RAPD and ISSR PCR products, respectively.

RESULTS AND DISCUSSION

In this experiment, single nodal segments were selected as explant source for *in-vitro* regeneration. The most prominent challenge faced in initiation stage of any *in-vitro* regeneration protocol is contamination by bacteria and fungi. Various chemicals are used to disinfect the explant such as fungicide, sodium hypochlorite, 70% ethanol, mercuric chloride, etc. It was also noted that natural environmental status sometimes contribute to *in-vitro* contamination rate such as collection of explant during rainy season greatly increased *in-vitro* contamination rate by upto 60%. Pre-treatment with 70% ethanol and 0.1% sodium hypochlorite for 10 minutes helped eliminate contamination.

Table 1. Effect of WPM supplemented with different concentration of cytokinin (BAP and Kinetin) on bud break percentage where maximum effect was observed with 3 mg/l BAP.

Media	Cytokinin	Concentration (mg/l)	Bud break %
WPM	-	0.0	50
	BAP	1.0	60
WPM		2.0	68
		3.0	74
		4.0	72
WPM	Kinetin	1.0	50
		2.0	55
		3.0	58
		4.0	55

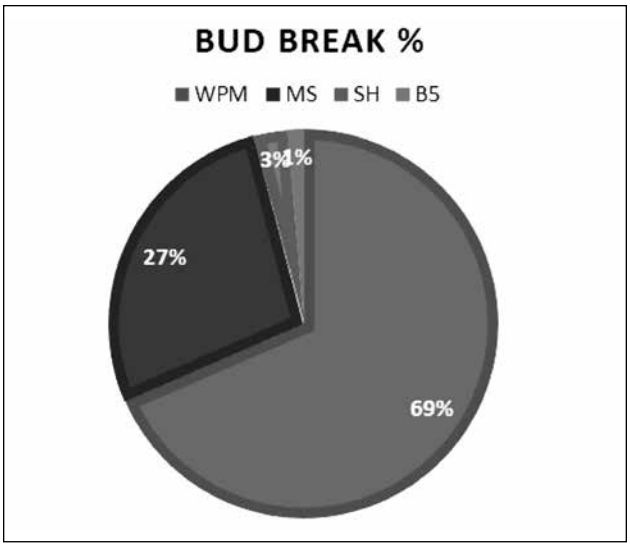

Fig. 1. The effect of different basal tissue culture media (WPM, MS, SH and B5) on percentage of bud break in *P. granatum* cv. Phule Arakta.

Out of the different basal tissue culture media tested, MS and WPM media showed 20% and 50% bud break, respectively (Fig. 1). Since WPM showed good result, it was used for further regeneration experiment. This suggests that WPM contains essential components required by pomegranate cv. Phule arakta for its regeneration.

The effect of WPM supplemented with BAP and kinetin on bud break percentage and number of days taken was studied where WPM supplemented with 3mg/l BAP gave the highest percentage of bud break (74%) in 8 days. A reduction in bud break was observed when higher concentration of BAP was added. Out of the two cytokinins studied, BAP was found to be more suitable than kinetin (Table 1).

BAP was more effective in inducing bud break compared to kinetin. Hence BAP was used for carrying out further studies for obtaining shoot proliferation. The effect of BAP at different concentrations of 0, 1, 2 and 3 mg/l was studied for shoot proliferation of pomegranate as noted in Fig. 2. It was observed that different concentrations showed significant effect on number of shoots and nodes formed as well as the average length of the shoots formed (Fig. 2). The medium supplemented with 2mg/l BAP exhibited maximum number of shoots (5.1±0.42) and nodes (8±0.8) [Fig. 2(A)]. This concord with the findings of Naik *et al.*, (10) in which BAP was more effective than

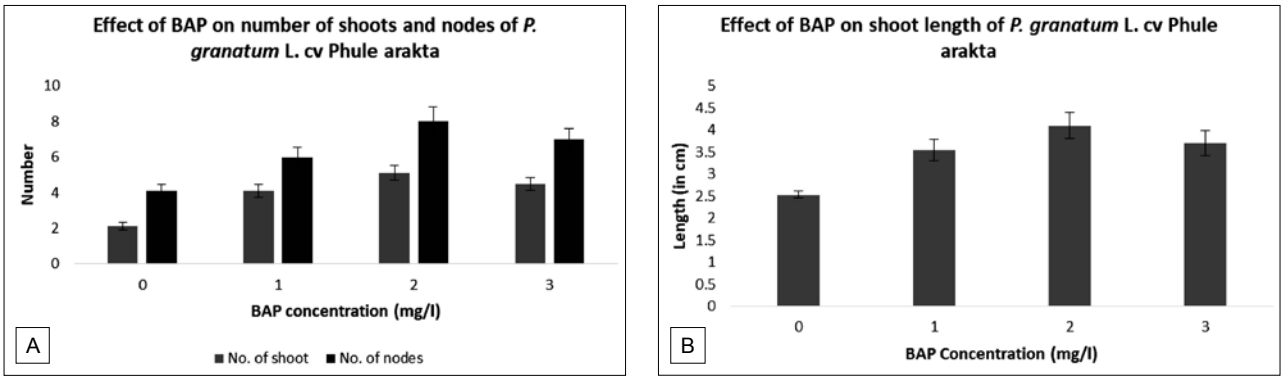

Fig. 2. Effect of different concentration of BAP on shoot proliferation of *P. granatum* L. cv. Phule Arakta. (A) Highest number of shoots and nodes were obtained in cultures grown in WPM supplemented with 2mg/l BAP. (B) The highest average length was obtained when WPM medium was fortified with 2mg/l BAP which declined at 3 mg/l BAP concentration.

Table 2. Effect of different concentration of adjuvants (silver nitrate, spermidine, adenine sulphate) in WPM supplemented with 2mg/l BAP on shoot proliferation of *P. granatum* L. cv. Phule Arakta.

Media	Adjuvant	Conc.(mg/l)	Number of shoots	Number of nodes	Length (cm)
WPM+2mg/l BAP	Silver Nitrate	0.0	5.0±0.4	8.0±0.8	4.1±0.28
		0.5	5.5±0.5	9.0±0.9	4.43±0.26
		1.0	7.0±0.5	10.0±0.9	4.6±0.28
		1.5	6.0±0.5	9.1±0.9	4.6±0.28
WPM+2mg/l BAP	Spermidine	0.0	5.0±0.4	8.0±0.8	4.08±0.28
		0.5	5.4±0.5	8.1±0.8	4.14±0.25
		1.0	5.6±0.5	8.8±0.9	4.13±0.25
		1.5	5.7±0.5	8.4±0.8	4.18±0.24
WPM+2mg/l BAO	Adenine sulphate	0.0	5.0±0.4	8.0±0.8	4.1±0.28
		0.5	5.1±0.5	8.3±0.7	4.1±0.29
		1.0	5.1±0.5	8.3±0.8	4.18±0.27
		1.5	5.1±0.5	8.2±0.8	4.23±0.26

kinetin and shoot development increased with the increase of BAP.

The addition of adjuvants such as Activated Charcoal (AC), PVP, silver nitrate, spermidine and adenine sulphate have shown significant effect on shoot production potential of *in-vitro* cultures (Giridhar *et al*., 4; Thomas, 15). The effect of adjuvants silver nitrate, adenine sulphate and spermidine was checked at different concentrations (0, 0.5, 1 and 1.5 mg/l). Significant difference was observed in the number of shoots and nodes when adjuvant silver nitrate was added at concentration of 1 mg/l (Table 2).

This resulted in maximum 7±0.5 number of shoots and 10±0.9 nodes, which is more than that obtained using only BAP at 2 mg/l concentration. However, no remarkable effect was observed while supplementing media with adenine sulphate and spermidine on both shoot number and number of nodes. There was no significant effect on shoot length when medium was supplemented with silver nitrate, adenine sulphate and spermidine (Table 2).

Pomegranate has high phenolic content resulting in oxidation, leaching and browning of culture media which causes necrosis of the explants. In order to overcome this problem, PVP, an absorbent was added at 250 mg/l to the multiplication media with no visible browning even after 3 weeks of inoculation. Subsequent sub-culturing after every 4 weeks also helped curb the problem of media browning. The new shoots developed were excised and cultured in the same media after every 4 weeks for upto 7th sub-culture cycle. The maximum number of shoots was obtained in the 6th sub-culture giving on average 9.1±0.36 shoots, 12.9±0.37 nodes and 64.9±0.99

leaves (Fig. 3). After the 6th sub-culturing cycle, a decline in shoot number, number or nodes and leaves was observed in the 7th cycle. This implies that sub-culturing upto 6th cycle gives the maximum shoot proliferation (Fig. 3). It is estimated that approximately, 117 (9x13) plantlets can be obtained by performing sub-culturing upto 6th cycle.

The results of *in-vitro* rooting induced by using auxins NAA and IBA are depicted in Table 3. Two types of experiments were carried out: one in which different concentrations (0, 1, 3 and 5mg/l) of IBA and NAA were included in WPM media and the other was shock treatment in which cultures were dipped in 100mg/l IBA and NAA and inoculated onto basal WPM media.

93% of explants produced rooting in 20 days when given shock treatment by dipping in 100mg/l NAA as compared to 79% in 26 days using IBA (Table 3). The best rooting percentage of 93% with average root number 5.7±0.3 and root length of 5.9±0.1 cm was obtained using shock treatment by NAA. IBA dipped cultures showed lesser percentage of rooting and took longer time to initiate rooting. The rooting percentage, root length and root formed per explant was more in NAA treated explants as compared to those treated with IBA (Table 3). As opposed to previous findings in which cultures inoculated in media containing auxins showed rooting in 10-15 days (Naik *et al*., 10), root initiation in media containing auxin NAA (5 mg/l) was observed in 29 days in our

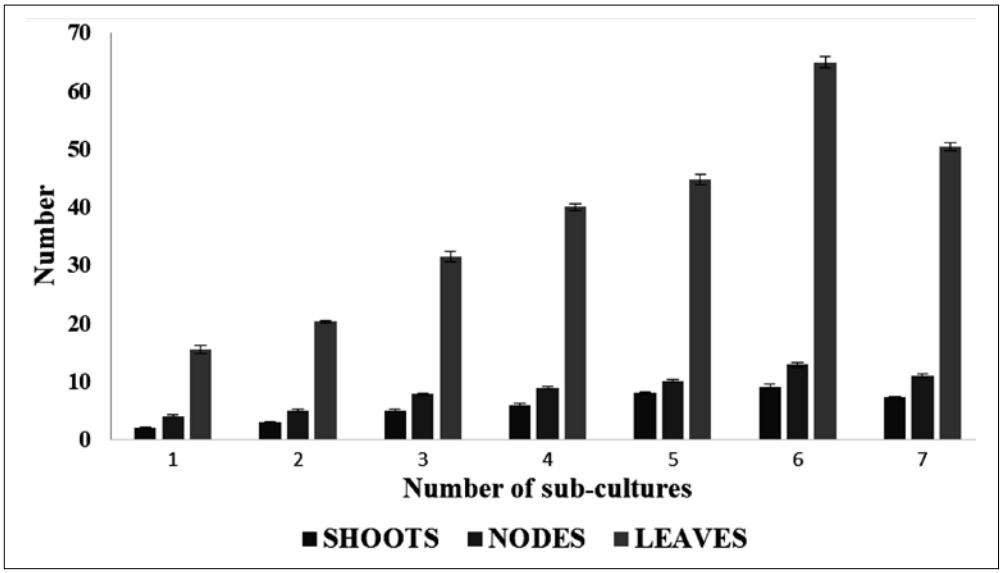

Fig. 3. Effect of sub-culturing carried out at interval of 4 weeks on *in-vitro* shoot proliferation of *P. granatum* L. cv. Phule Arakta.

Table 3. Effect of auxins (IBA and NAA) at different concentration in WPM as well as shock treatment with 100mg/l concentration on rooting of *P. granatum* L. cv. Phule Arakta.

Treatment number/ explant	Conc. (mg/l)	No. of days taken to obtain rooting	% rooting explant	Root length (cm)	Root
WPM+IBA	0	-	0.0	0.0	0.0
WPM+IBA	1	-	19.3±0.7	0.8±1.5	1.3±0.3
WPM+IBA	3	-	49.0±1	2.0±0.1	2.7±0.3
WPM+IBA	5	33	57.7±1.5	3.8±0.1	3.7±0.3
Dipped in IBA	100	26	79.0±0.6	5.3±0.1	4.7±0.3
WPM+NAA	0	-	0.0	0.0	0.0
WPM+NAA	1	-	28.3±1.7	0.9±0.1	1.7±0.3
WPM+NAA	3	-	61.0±0.6	3.0±0.1	3.0±0.6
WPM+NAA	5	29	74.7±0.3	4.8±0.1	4.7±0.3
Dipped in NAA	100	20	93.0±1.5	5.9±0.1	5.7±0.3

study. Significant difference using two way ANOVA studies (α=0.05) was observed in rooting percentage, number of roots formed per explant and root length between treatments (different concentrations) using IBA and NAA (Table 3). This indicates that there is a marked difference in the rooting response when the *in-vitro* regenerated shoots were treated with IBA and NAA in which shoots inoculated in 5mg/l NAA resulted in a higher percentage of rooted plantlets (74.7%) as well as root length (4.8±0.1 cm) and root number per explant (4.7±0.3) as compared to 5mg/l IBA in which on an average 57.7% rooted plantlets were observed with an average of 3.8±0.1 cm in root length and 3.7±0.3 root number per explant. Similar observations were made when shock treatment using 100mg/l NAA and IBA was given. Two sub-culture cycles in which rooted cultures were transferred onto fresh media every three weeks was enough to get a sturdy root system.

The plantlets with well-developed roots were then transferred onto potting mixture containing autoclaved cocoa peat and perlite in 1:1 ratio and exhibited 100 % survival rate after 30 days (Fig. 4).

Progenies or plantlets raised through tissue culture are expected to be genetically uniform with the mother plant. However, genetic variability or somaclonal variation is seen in plants produced through tissue culture and particularly common in plants regenerated through callus culture. These can, however, be minimized by using axillary induction system, reducing number of sub-culturing, avoiding usage of 2,4- Dichlorophenoxyaceticacid (a hormone that induces genetic variation) and avoiding long term cultures (Bhatia & Sharma, 3). RAPD and ISSR makers have been reported to be employed for assessing genetic fidelity of micropropagated of *Musa acuminata* (Lakshmanan *et al*., 9), *Dendrocalamus strictus* (Roxb.) (Goyal *et al*., 5) and *Simmondsia chinensis* (Kumar *et al*., 8). In this study, RAPD and ISSR markers were employed to check for somaclonal variations in the *in-vitro* raised *Punica granatum* L. plantlets. Out of the 10 RAPD and 5 ISSR primers screened for reproducibility in P. granatum L. cv. Phule arakta, all 10 RAPD primers produced clear and scorable amplified products, whereas only 4 out of 5 ISSR primers were reproducible. The 10 RAPD primers produced a total of 55 scorable bands ranging from 250 bp to1500 bp and the 4 ISSR primers produced 19 bands of band sizes ranging from 400bp to 1700bp (Table 4). Variation in number of bands ranging from 2 (OPA-14) to 7 (OPM-20, OPD-7, OPC-13, OPC-08) with an average of 5 bands per RAPD primer and 3 (UBC-817) to 6 (UBC-834) with average of 5 bands per ISSR primer were observed (Table 4).

The screening of somaclonal variation was done using these reproducible RAPD and ISSR primers, all of which generated monomorphic bands (Fig. 5) which confirms genetic fidelity of *in-vitro* raised *P. granatum* L. plantlets. A total of 70 monomorphic bands from RAPD primers OPC-13 and OPM-20;

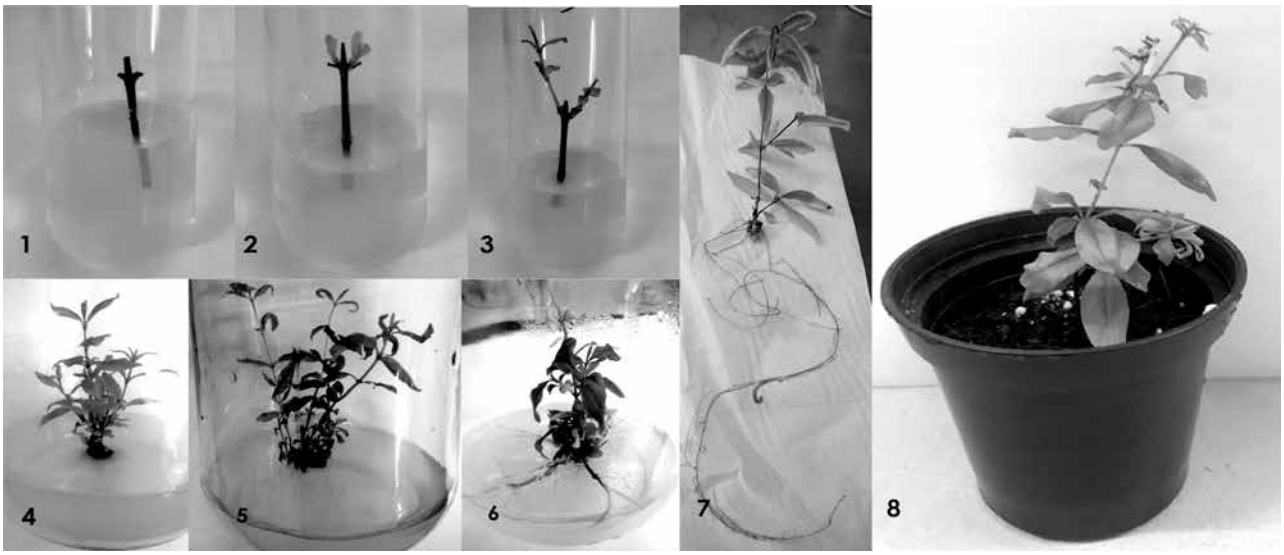

Fig. 4. Different stages of development of *in-vitro* raised *P. granatum* L. cv. Phule Arakta. (1) Nodal segment as explant; (2) Bud break and formation of young leaves in WPM; (3) Elongation of axillary shoots; (4) Multiple shoot proliferation in proliferating media; (5) Healthy elongated shoots after sub-culturing; (6) Induction of roots in rooting media; (7) *In-vitro* plantlet with healthy roots; (8) Hardening of rooted plantlets in autoclaved cocoa peat and pearlite.

Table 4. List of RAPD and ISSR primers (Lakshmanan *et al*., 9) and PCR amplicons generated in *in-vitro* raised *Punica granatum* L. cv. Phule Arakta.

Sl. No.	Primer ID	Primer Sequence (5'-3')	G+C Content (%)	No. of scorable bands	No. of monomorphic bands	Band size (bp)
RAPD						
1	OPA-04	AATCGGGCTG	60	4	4	400-1000
2	OPA-14	CTCGTGCTGG	70	2	2	400-600
3	OPA-20	GTTGCGATCC	60	5	5	350-1000
4	OPC-08	TGGACCGGTG	70	7	7	300-1600
5	OPC-09	CTCACCGTCC	70	6	6	250-1400
6	OPC-13	AAGCCTCGTC	60	7	7	380-1300
7	OPD-07	TTGGCACGGG	70	7	7	350-420
8	OPD-08	GTGTGCCCCA	70	5	5	450-1200
9	OPM-20	AGGTCTTGGG	60	7	7	350-900
10	OPM-18	CACCATCCGT	60	5	5	300-850
ISSR						
1	UBC-811	GAGAGAGAGAGAGAGAC	53	5	5	500-2000
2	UBC-817	CACACACACACACACAA	47	3	3	350-800
3	UBC-820	GTGTGTGTGTGTGTGTT	47	0	0	0
4	UBC-826	ACACACACACACACACC	53	5	5	400- 1100
5	UBC-834	AGAGAGAGAGAGAGAGYT	44 to 50	6	6	450- 1200

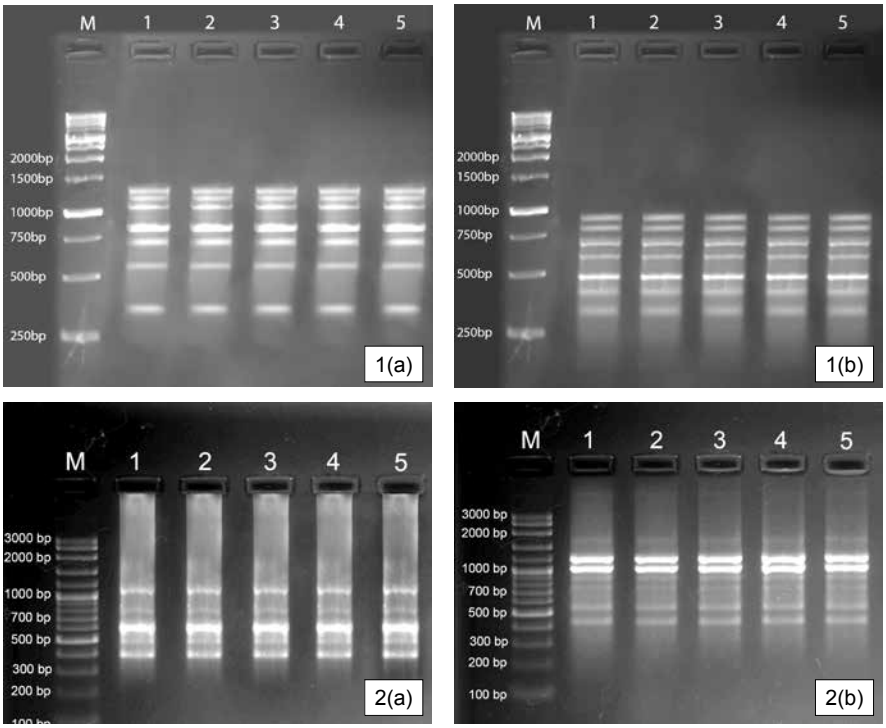

Fig. 5. Genetic fidelity testing using molecular markers RAPD and ISSR between mother plant (Lane 1) and *in-vitro* progenies (Lane 2: 3rd sub-culture, lane 3: 6th sub-culture, Lane 4: randomly selected, Lane5: rooted culture) of *P. granatum* L. cv. Phule Arakta. **1(a&b)**. RAPD primers OPC-13 (1a) and OPM-20 (1b) generated polymorphic bands with reference 1Kb DNA ladder (M) (GeneDireX® 1Kb DNA Ladder RTU). **2(a&b).** ISSR primers UBC-826 (2a) and UBC-834 (2b) resulted in uniform banding pattern with 100bp DNA ladder as reference (M) (100bp Plus DNA Ladder (0.1µg/µl) vivantis).

and 55 monomorphic bands from ISSR primers UBC-811 and UBC-826 were generated among the mother plant and *in-vitro* samples analyzed (Plantlets from 3rd, 6th sub-culture, a random sample and rooted plantlet). This confirms that genetic integrity was maintained when micropropagation of *P. granatum* cv. Phule Arakta was carried out through nodal segment.

In conclusion, this study has resulted in development of an efficient micropropagation protocol for the highly valuable edible tree species, *Punica granatum* L. cv. Phule Arakta in which no somaclonal variation between mother plant and *in-vitro* raised plantlets of *P. granatum* L was observed using RAPD markers. Through this robust micropropagation protocol, a maximum of 117 plantlets can be obtained in the 6th sub-culture cycle from a single node. Thus, micropropagation through nodal segment multiplication is an effective and preferred method for producing true-to-type progenies *in-vitro*. The RAPD and ISSR primers reported in our research is, for the first time utilized for performing genetic fidelity testing for *P.granatum* L. cv. "Phule Arakta".

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